In vitro selection of microspore derived embryo genotypes based on molecular marker and oil quality analysis in oilseed rape (*Brassica napus* L.)

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Abstract

Microspore culture is frequently applied for the immediate regeneration of homozygous doubled haploid plants in oilseed rape. From the regenerated microspore derived embryos (MDE) usually only a smaller subset are used for plantlet regeneration and cultivation in the greenhouse until seed harvest, without any knowledge about their quality traits and agronomic performance. The random selection of MDE implies that valuable rare recombinant genotypes may be discarded at an early stage of *in vitro* culture. We report here on the development of a simple protocol for simultaneously extracting lipids (for oil quality analysis) and the isolation of DNA (for marker assisted selection) from single cotyledons dissected from MDE under aseptic conditions, thus keeping the rest of the embryo *in vitro* for plantlet regeneration. Neither the fatty acid extraction nor the transmethylation with sodium methylate at high pH did interfere with subsequent DNA isolation. The feasibility of the protocol was tested using MDE from a cross segregating for two linked transgenes, *fae*1 and *pls*C, affecting the fatty acid composition. Multiplex-PCR was performed with specific PCR-primers for the *pls*C-gene and with locus specific primers for a resident single copy *fad*2 gene. The amplification of the *pls*C gene showed a 1:1 segregation expected for a single copy transgene in a segregating doubled haploid population. The early identification of the 50% MDE genotypes carrying the desired transgenes along with a high expression of the trait allows their early selection for plantlet regeneration.

Key words: Rapeseed, *in vitro*, MDE, *pls*C-gene, molecular marker

Introduction

Anther and isolated microspore culture are applied to regenerate androgenic haploid embryos and plants in different crop species, which after successful colchicine induced diploidization result in the production of completely homozygous genotypes. In oilseed rape (*Brassica napus* L.) this method has gained considerable importance in breeding programmes. Presently, hundreds to thousands of microspore derived embryos (MDE) can be obtained from a single microspore preparation. Normally, only smaller fractions (200-300) of these MDE are sub-cultured *in vitro* to regenerate plantlets which are then transferred to the greenhouse for seed production. The subset of MDE genotypes used for plantlet regeneration represents a random sample of the total MDE without any information about their traits. Therefore, a large number of undesired genotypes are carried through the laborious and time consuming process of plantlet regeneration and seed production in the greenhouse. It also implies that valuable rare recombinant genotypes may be discarded at an early stage of *in vitro* culture. Any method that could be applied to determine useful agronomic or seed quality traits at an early stage of *in vitro* culture definitely would help to increase the frequency of valuable genotypes among the total number of regenerated MDE. The application of an early *in vitro* selection system is limited to those traits which can be rapidly analysed and for which a close correlation between the MDE and the seeds from the regenerated plants exists. Furthermore, phenotypic results may be influenced by MDE genotype × environment interactions, i.e. *in vitro* culture conditions may affect the fatty acid composition of storage lipids in MDE genotypes differently. Additional and better results should be obtained if marker assisted PCR based selection could be applied at the same time. The objective of the present study was to investigate the possibility of analysing the fatty acid composition simultaneously to the isolation of DNA suitable for PCR amplification using single dissected cotyledons of MDE of oilseed rape.

Materials and Methods

The homozygous transgenic resynthesized high erucic acid rapeseed line RS306 (TNKAT), carrying a single T-DNA with the chimeric *Bn-fae*1.1-*pls*C genes, both under control of the seed specific napin promotor (Han *et al.* 2001) were crossed to the high erucic acid, low polyunsaturated fatty acid winter rapeseed line 6575-1 (Sasongko and Möllers 2005). F_1-plants and TNKAT parental plants were used for microspore culture which was performed as described in Iqbal *et al.* (1994). Three weeks after microspore culture, MDE were transferred to fresh medium at a density of 10 embryos in Petri dishes (6 cm) with 7 ml of NLN medium containing 13% sucrose to allow for storage lipid accumulation and to prevent germination. Three weeks after transfer, embryos were well developed (Fig. 1) and a single cotyledon was dissected. The remaining embryo was cultured on solid MS medium for plantlet regeneration. The dissected cotyledon was dried overnight at 60° C in 1.5 ml Eppendorf tubes. Lipid and subsequent DNA extraction from the dried MDE cotyledons were done according to Nath *et al.* (2006), following a modified protocol initially described by Ishizawa *et al.* (1991). Fatty acids were analysed by gas liquid

chromatography (GLC) following the method described by Albrecht *et al.* (1995).

Suitable PCR primers for amplification of the reporter *npt*II-gene and *plsC*-gene (Ld-LPAAT from *Limnanthes douglasii*) were obtained from www.lag-gentechnik.de/dokumente/ok_laurical_raps.pdf (Site last visited 29.11.2006). Primer sequences were NPT-F: 5′-ATCGGGAGCGGCGATACCGTA-3′, NPT-R: 5′-GAGGCTATTCGGCTATGACTG-3′; LPAAT-F: 5′-CCGCAACAGGAGACAACTAAA-3′ (nt 36, Genebank: X83266) and LPAAT-R 5′-TATTGGGAGATGTGACTGAAG-3′ (nt 639). For positive control for the presence of DNA in the PCR mix, a locus specific primer pair for the *fad*2 gene of the *Brassica napus* A-genome (*B. rapa*) was used (Spiekermann 2003). Primer sequences were FAD2A-F 5′-ATGGGTGCAGGTGGAAGAATG-3′ and FAD2A-R 5′-CAGTTTCTTCTTTGCTTCATAAC-3′. The Multiplex PCR mixture was prepared by mixing in 16.25µl of HPLC water, 2.5 µl of 10xPCR buffer ($MgCl_2$ free), 1.5 µl of $MgCl_2$ (2mM), 0.5 µl of dNTP's (0.4mM), 1.0 µl of each FAD2 primer (0.4mM), 0.5µl of each NPTII primer (0.12mM), 0.25 µl of Taq polymerase (1.25 units) for *npt*II-gene and for *plsC*-gene following Nath *et al.* (2006), respectively. To this, 1 µl of the isolated DNA was added to give a total of 25 µl for a single PCR reaction. The amplification was performed in a Biometra Tgradient thermal cycler (Biotron, Germany) and PCR reaction products were separated and stained as described in Nath *et al.* (2006).

Results and Discussion

The extraction of lipids and DNA from a single MDE cotyledon requires that the embryos have a relatively large size. This was achieved in the present study by sub-culturing the MDE after 3-4 weeks of culture initiation in fresh medium with 13% sucrose. Thereby, the high sucrose content allowed storage lipid biosynthesis and prevented germination of the embryos during this period. Figure 1 shows the development of MDE from week 2-6 after culture initiation and the relative size in comparison to a single rapeseed kernel. At week 6, MDE are sufficiently large to allow easy dissection of one cotyledon for lipid and DNA extraction, analog to the half seed method developed by Thies (1971) to select among segregating F_2 seeds for fatty acid composition.

Fig. 1. Development of microspore derived embryos from week 2 to week 6 after culture initiation and relative size in comparison to a seed of oilseed rape (*Brassica napus* L.).

The dry weight of single dissected cotyledons used for lipid and DNA extraction ranged from 4 – 18 mg (Tab. 1). From this it was possible to extract a sufficient amount of oil for reliable and reproducible fatty acid analysis using a gas chromatograph. The total peak area values for the sum of fatty acids ranged between 87 – 209 with a mean of 138, which gave a good resolution of the fatty acid profile. The dried pellet obtained after oil extraction and transmethylation was used for DNA isolation and subsequent PCR amplification. DNA extraction was insufficient and PCR amplification was inconsistent when 6M NaI buffer was used (Tab. 1). By reducing the molarity of NaI to 4M and 2M in the extraction buffer, the consistency of DNA amplification was improved with 2M NaI giving consistent results. However, DNA isolation was not successful in all cases (see Tab. 1: 20 out of 22 MDE; = 91%), indicating the necessity of an internal control system. This was realized in the present study by performing multiplex PCR, including PCR primers for the resident, single copy *fad*2 locus (oleic acid desaturase) of the *Brassica* A-genome. This allowed the identification of samples which did not contain DNA in sufficient quantity or quality for successful PCR amplification, e.g. samples 11 and 12 in Figure 2.

Table 1. Consistency of PCR amplification of DNA by different extraction methods using detached cotyledons of microspore derived embryos (MDE) of *B. napus* (Nath *et al.* 2006)

Extraction buffer	Cotyledons from MDE		
	Mean dw mg	Range mg	Consistency of amplification
6 M	9.9	6 – 15	6/18
4 M	6.8	4 – 10	10/18
2 M	9.5	5 – 18	20/22

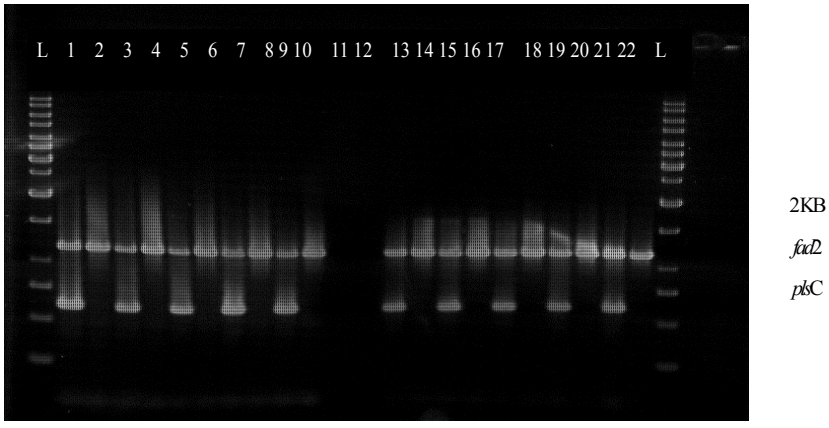

Fig. 2. Multiplex-PCR of a 603bp fragment of the *pls*C gene together with internal control amplification of the 1.1kb *fad*2 gene sequence using DNA extracted from 22 MDE (Lanes 1-22) segregating for the *pls*C gene.

In the segregating MDE population the *pls*C-gene was amplified only in 50% of the genotypes, confirming the expected 1:1 segregation of a single copy transgene in a doubled haploid MDE population (see example in Fig. 2). Above mentioned segregation was also confirmed by the PCR amplification of the *npt*II gene using multiplex-PCR. The storage lipids isolated from the cotyledons of the MDE showed clear differences in the content of erucic acid and polyunsaturated fatty acids, however, transgenic MDE in general did not show an increased erucic acid content (data not shown). Figure 3a,b shows that among the segregating MDE there are some MDE which have a higher 22:1 and an about equal 18:1, but a reduced PUFA content compared to the transgenic parental MDE (TNKAT). This indicates that the reduction in PUFA content may have led to an increased 22:1 content.

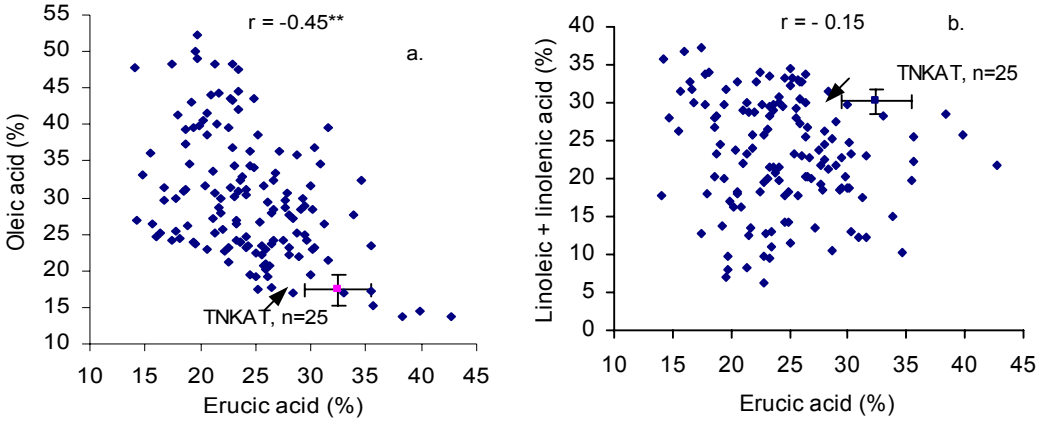

Fig. 3a-b. Fatty acid compositions of MDE derived from a cross between transgenic high erucic acid and non-transgenic high erucic acid rapeseed. ** indicates significance at P= 0.01 probability.

Doubled haploid lines regenerated from MDE are currently being cultivated in the greenhouse to harvest seeds. A comparison of the fatty acid profile of the MDE with the fatty acid profile of the seeds from the derived plants will give clear results about the effect of the *fae*1-*pls*C-transgene copy on the fatty acid profile.

Conclusions

In the present study, it was shown that neither the fatty acid extraction nor the transmethylation with sodium methylate at high pH did affect subsequent DNA isolation in sufficient quantity and quality for PCR reactions. Nevertheless, the multiplex PCR approach using locus specific primers for the resident *fad*2-gene provides an easy control system for the presence of DNA. The segregation of a single gene/transgene in a DH population is the simplest example (1:1 segregation). If two or three genes are segregating, than only 25% and 12.5% of the MDE carry the positive alleles, respectively. Considering the polygenic inheritance of many agronomic and seed quality traits, the early detection of those MDE genotypes having positive alleles by marker assisted selection enables their early identification and preferential regeneration to plantlets. Hence, much labour and greenhouse space is saved during plantlet transfer to soil and their cultivation in the greenhouse.

Acknowledgements

UKN and MCMI gratefully acknowledge a Research Fellowship from DAAD, Bonn, and a travel grant from the NSF, Sri Lanka. Thanks to Dr. Margrit Frentzen (RWTH Aachen, Institute for Biology I, Unit of Botany) for providing seeds of the transgenic line RS306 (TNKAT). The technical assistance of Uwe Ammermann (GLC analysis) and Rosemarie Clemens (PCR) is gratefully acknowledged.

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